phosphate buffer, pH 7.4, 30 min). The electrochemical measurements were performed by using an electrochemical impedance analyzer (EG&G, model 1025) and potentiostat (EG&G, model 283) connected to a computer (EG&G Software Power Suite 1.03 and no. 270/250 for impedance and cyclic voltammetry, respectively). All the measurements were performed in 0.1M phosphate buffer solution, pH 7.0, at room temperature. When needed, oxygen was removed from the solution by passing Ar above the cell. The Faradaic impedance measurements were performed in the frequency range of 100 mHz to 50 kHz in the presence of 10 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1 mixture) as a redox probe and upon biasing the working electrode at $E = 0.175$ V. The electrochemically induced biochemiluminescence was measured with a light detector (Laserstat, Ophir) linked to an oscilloscope (Tektronix TDS 220). The light detector was connected to the electrochemical cell by an optical fiber and a potential corresponding to $E = -0.7$ V was applied on the working electrode. The background electrolyte solution was equilibrated with air and included luminol (1×10^{-6}M), and HRP (1 mg mL^{-1}). The electrochemically induced precipitation of the insoluble material (**5**) was performed upon application of a potential that corresponded to $E = -0.7$ V on the working electrode in 0.1M phosphate buffer, pH 7.0. The electrolyte solution was equlibrated with air and included 4-chloro-1-naphthol (**4**; 1×10^{-3}M) and HRP (1 mg mL^{-1}).

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An Electrochemical Probe of DNA Stacking in an Antisense Oligonucleotide Containing a C3′-*endo*-Locked Sugar**

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The sensitivity of charge transport chemistry to base stacking[1–5] provides the foundation for applications of DNA charge transport that probe nucleic acid structure, particularly those utilizing electrochemistry experiments on DNA films.[6–13] In these experiments, DNA oligonucleotide duplexes modified with a thiol linker are self-assembled on a gold electrode surface. A redox-active intercalator, such as methylene blue, bound to the close-packed DNA films is electrochemically reduced and the reduced intercalator is used as a catalyst for the reduction of a species diffusing in solution outside of the DNA film (usually ferricyanide). Once re-oxidized by ferricyanide, methylene blue is available for subsequent electrochemical reduction and the catalytic cycle continues.[9,10] The electrochemical reduction of methylene blue takes place via charge transport through the DNA base stack, and thus perturbations in base-pair stacking are repeatedly interrogated in this assay, rendering the electrocatalytic assay exquisitely sensitive to even the smallest disruptions in π stacking. Using this technique, we have detected all single base mismatches as well as several common DNA base damage products.[10] Base-stacking perturbations are also detected within DNA/RNA hybrid duplexes.[12] Furthermore, the electrochemical reduction of DNA intercalators bound to DNA-modified electrodes has been used to monitor DNA–protein interactions.[13] Since this chemistry is extremely sensitive to very small changes in DNA base-pair stacking, we may exploit this assay more generally in probing

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local perturbations in nucleic acid structure and stacking. Herein we describe the application of electrocatalysis at DNA-modified electrode surfaces to probe stacking in antisense oligonucleotides (AON) containing a 3′-*endo*-locked conformation. Figure 1 illustrates different duplex films that have been probed.

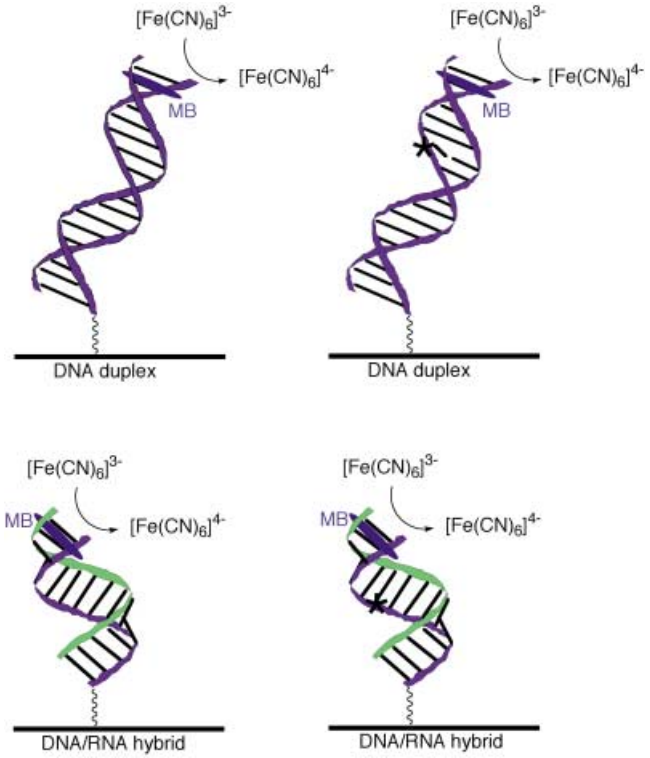
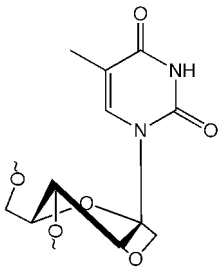


Figure 1. Schematic illustration of our electrocatalytic method to directly measure stacking in oligonucleotide duplexes. Here we probe the stacking of **T** (*) within a DNA (purple) scaffold and within a DNA(purple)/RNA(green) hybrid scaffold. Oligonucleotide duplexes are tethered to alkanethiol linkers and self-assembled onto Au electrode surfaces for use in electrochemical assays. Stacking is probed in a DNA-mediated charge transport reaction to reduce methylene blue (MB) that serves as a catalyst to reduce ferricyanide in solution. If stacking is disrupted, methylene blue is not efficiently reduced and less charge accumulates over the course of the catalytic assay.

Hybrid duplexes formed by AON containing one North-form (3′-*endo*) locked 1-(1′,3′-*O*-anhydro-β-D-psicofuranosyl) thymine block **T** paired with RNA (AON/RNA heteroduplex) are completely inactive to cleavage by RNase H in four nucleotides to the 5′ side of the conformationally constrained nucleotide.[14] In fact, a single cleavage site can be engineered in a AON/RNA hybrid at the site of **T** incorporation.[15] This protection from cleavage is proposed to result from the formation of a local RNA/RNA fully A-form duplex conformation in the vicinity of **T** as opposed to a mixed DNA/RNA hybrid conformation. This result underscores the importance of knowledge of local conformation for antisense design and recruitment of RNase H. Furthermore, these studies lead to the suggestion that incorporation of a 3′-*endo*-type-locked nucleotide (typical for A-form duplexes) into a nucleic acid duplex leads to formation of a *local* A-form duplex which, if true, could lead to a *junction* in base-pair stacking.



T

Structural studies have also been performed on oligonucleotide duplexes containing **T** moieties.[16] Circular dichroism (CD) spectra show that there is more distortion in a DNA/DNA duplex containing **T** than the corresponding DNA/RNA hybrid duplex. Furthermore, although a T_m drop of 6 °C/**T** incorporation is observed, the Watson–Crick hydrogen bonding remains intact as is evident from ^{1}H NMR studies. This suggests that **T** is hydrogen-bonded in the duplex base stack and the T_m drop is likely the result of a change in the stacking geometry of the duplex, not loss of hydrogen bonding as with mismatches in DNA. Given the differing behavior in a DNA/DNA versus DNA/RNA duplex, it seems likely that this change in stacking geometry of the duplex is orchestrated by the sugar conformation.

These structural data coupled with the RNase H cleavage protection studies suggest that **T** may introduce a junction in the base-pair stacking of DNA. However none of these methods probe the base stacking of DNA directly. Charge transport through DNA, however, does provide a direct assay of base-pair stacking. Thus to determine whether **T** disrupts base-pair stacking within a DNA scaffold and within a DNA/RNA hybrid scaffold, we incorporated **T** into DNA-modified electrode surfaces and monitored the electrocatalytic efficiency in these films (Figure 1). Electrode surfaces modified with duplexes **1**–**12** (Table 1) were interrogated in electro-

Table 1. Oligonucleotides probed in this study.[a]

1	SH-5′GAAGAAAAAATGAAG CTTCTTTTTTACTTC
2	SH-5′GAAGAAAAAATGAAG CTTCT**T**TTTTACTTC
3	SH-5′GAAGAAAAAATGAAG CTTCTTT**T**TTACTTC
4	SH-5′GAAGAAAAAATGAAG CTTCTTTTT**T**ACTTC
5	SH-5′GAAGAAAAAATGAAG CT**T**CTTTTTTACTTC
6	SH-5′GAAGAAAAAATGAAG CTTCT**C**TTTTACTTC
7	SH-5′GAAGAAAAAATGAAG CTTCT**A**TTTTACTTC
8	SH-5′GAAGAAAAAATGAAG CTTCT**G**TTTTACTTC
9	SH-5′AGTACAGTCATCGCG TCATGTCAGTAGCGC
10	SH-5′AGTACAG**T**CATCGCG TCATGTCAGTAGCGC
11	SH-5′AGTACAGTCATCGCG tcatgtcagtagcgc[b]
12	SH-5′AGTACAG**T**CATCGCG tcatgtcagtagcgc

[a] SH-5′ stands for $SH(CH_2)_2CONH(CH_2)_6NHCO$ attached to the 5′ OH of the DNA single strand. [b] Lowercase lettering indicates RNA.

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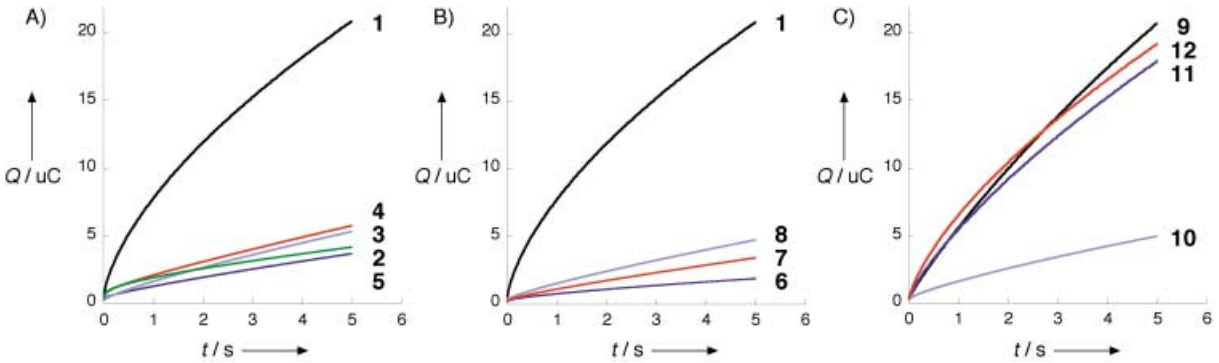

Figure 2. Electrochemical results for **T** incorporated into DNA-modified surfaces. Sequences **1**–**12** were self-assembled on clean gold electrode surfaces and used in electrocatalysis experiments.[7–10] Chronocoulometric analyses took place at −350 mV with 0.5 μM methylene blue (MB) as the intercalated catalyst and 2.0 mM $[Fe(CN)_6]^{3-}$ as the solution-borne substrate. A) Chronocoulometry plot (Q = charge) showing the results for **T** incorporated at a variety of positions within the duplex (sequences **1**–**5**). Within a DNA/DNA duplex, **T** creates a stacking perturbation similar to a mismatch. B) Chronocoulometry plot showing the results for mismatches resulting in sequences **6**–**8**, compared to the perfectly complementary native sequence **1**. C) Chronocoulometry plot showing the results for **T** incorporated into DNA/DNA verses DNA/RNA modified surface, sequences **9**–**12**. Within a DNA/DNA duplex, **T** creates a stacking perturbation (**10**), however, within a DNA/RNA duplex, stacking is normal (**12**).

catalytic chronocoulometric analyses at −350 mV (vs. SCE) with 0.5 μM methylene blue (MB) as the intercalated catalyst and 2.0 mM $[Fe(CN)_6]^{3-}$ as the solution-borne substrate.[9,10]

Figure 2A shows the results of **T** incorporation into DNA/DNA duplexes (sequences **1**–**5**). In these DNA/DNA B-form duplexes, the current is attenuated, similarly to that in mismatch controls (Figure 2B). Thus these data suggest that incorporation of a 3′-*endo* constrained pentoribofuranose unit into a duplex where all other deoxyribose units are 2′-*endo* creates a *base stacking* perturbation.

We further explored this phenomenon by incorporating **T** into DNA/DNA homoduplexes versus DNA/RNA hybrid duplexes, as shown in Figure 2C. In the DNA/DNA homoduplexes (**10**) **T** again yields a diminution in integrated charge. However, upon incorporation of **T** into a DNA/RNA A-form heteroduplex (**12**), little diminution in integrated charge, consistent with little perturbation in stacking, is evident.

These electrochemical data are in excellent correlation with the Rnase H protection studies[14,15] and NMR results.[16] The electrochemical data suggest that ribose conformation of a nucleotide moiety dictates its stacking in a duplex. In B-form DNA/DNA duplexes, the sugar conformation is normally 2′-*endo*. Thus when a nucleotide constrained to a 3′-*endo*-type conformation is incorporated into the duplex, the associated base assumes typical A-form stacking (and possibly transfers this conformation to four other neighboring nucleotides[14]), which results in a stacking junction between the A- and B-form parts of the duplex. When **T** is incorporated into a hybrid duplex, where the conformation is between A- and B-form, but generally closer to A-form, this locked nucleotide is better accommodated by the neighboring nucleotides and does not lead to a stacking perturbation.

Thus, these observations suggest that the origin of the drive for helical order vis-a-vis disorder in determining the *local structure* of a nucleic acid duplex lies in the preferred nature of the sugar conformation, which can be designed, engineered, and enforced to orchestrate the helix-forming process. Importantly, these results illustrate the utility of DNA-mediated charge transport electrochemistry through DNA-modified surfaces as a tool for directly probing DNA base pair stacking.

Experimental Section

Materials: All DNA synthesis reagents were obtained from Glen Research. Methylene blue and potassium ferricyanide were purchased from Aldrich and used as received. **T** was synthesized and incorporated into DNA by using a previously described procedure.[17]

Preparation of DNA-modified surfaces: Thiol-modified oligonucleotides were prepared by using phosphoramidite synthesis as previously described. Thiol-terminated linkers were attached to single-stranded oligonucleotides, purified by HPLC, and hybridized to unmodified complements. These duplexes (0.1 mM) were then deposited on polycrystalline gold electrodes for 12–24 h, thoroughly rinsed with buffer, and used for electrochemical experiments.[6–13] These surfaces were characterized by cyclic voltammetry, ellipsommetry, radiolabeling of the duplexes, and atomic force microscopy. We have found that densely packed monolayers with a 45° orientation of the helical axis with respect to the gold surface are formed when no potential is applied to the electrode surface.[7]

Electrochemical measurements: Chronocoulometry was carried out anaerobically (bubbling argon) on 0.02 cm^2 gold electrodes using a Bioanalytical Systems (BAS) Model CV-50W electrochemical analyzer. Buffer and electrolyte conditions were 5 mM sodium phosphate buffer containing 50 mM NaCl, pH 7, ambient temperature. A normal three-electrode configuration consisting of a modified gold-disk working electrode, a saturated calomel reference electrode (SCE, Fisher Scientific), and a platinum wire auxiliary electrode was used. The working compartment of the electrochemical cell was separated from the reference compartment by a modified Luggin capillary. Potentials are reported versus SCE.

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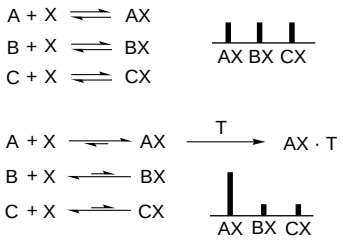
Enzymatic Generation and In Situ Screening of a Dynamic Combinatorial Library of Sialic Acid Analogues**

Roger J. Lins, Sabine L. Flitsch,* Nicholas J. Turner,* Ed Irving, and Stuart A. Brown

Dynamic combinatorial chemistry (DCC) is a rapidly emerging field which offers a possible alternative to the approach of traditional combinatorial chemistry (CC).[1] Whereas CC involves the use of irreversible reactions to efficiently generate static libraries of related compounds, DCC relies upon the use of reversible reactions to generate dynamic mixtures. The binding of one member of the dynamic library to a molecular trap (such as the binding site of a protein) is expected to perturb the library in favor of the formation of that member (Scheme 1). Comparison of the "perturbed" library with that generated in the absence of the trap should indicate which members of the library are interacting with the trap, which effectively offers in situ screening of the combinatorial library.

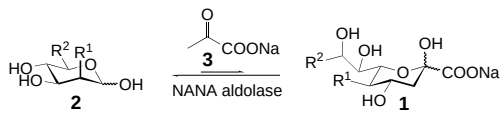
The DCC concept has already been proven through the elegant experiments by several research groups, including those of Lehn and Sanders.[1,2] However, significant experimental challenges remain before the method may be considered a practical complement to traditional CC. In particular, the in situ screening, which is such an attractive feature of DCC, demands the use of conditions amenable both to the library-formation and -trapping stages. If the trap is envisaged as a protein or other biomolecule, the system is limited to aqueous and near-physiological conditions, with which few covalent bond forming reactions are compatible. Thus, for example, Lehn and co-workers have employed both imine and disulfide exchange reactions in DCC experiments involving biomolecular traps,[2a] but to our knowledge no DCC experiment involving the formation of carbon–carbon bonds under physiological conditions has been performed.[3]



Scheme 1. The DCC concept: reversible reactions performed with a limiting amount of **X** generate a mixture of compounds **AX**, **BX**, and **CX**. The binding of **AX** to molecular trap **T** causes perturbation of the equilibria involving **A** and **X** to give overall amplification of **AX** at the expense of the other library members.

We believe that enzyme-catalyzed reactions, which are characteristically reversible under physiological conditions, are ideally suited to the generation of dynamic combinatorial libraries. Many enzymes with broad specificity (required for library diversity) are already commercially available, and the application of modern techniques in directed evolution may be expected to increase their number. The products of an enzyme-catalyzed reaction are usually stable compounds; simple removal or inactivation of the enzyme stops the reaction, thus reducing the dynamic mixture to a static library which may be analyzed directly, without the need for a derivatization step to freeze the product distribution. Herein we present the first demonstration of DCC using enzyme catalysis for the generation of a dynamic library.

In considering the application of enzyme catalysis to DCC, we were encouraged by the thermodynamic resolution of a dynamic mixture of aldol products by Whitesides and co-workers through the use of a broad-specificity aldolase to effect reversible formation of carbon–carbon bonds under mild conditions.[4] For the current investigation we chose a related enzyme, *N*-acetylneuraminic acid aldolase (NANA aldolase, EC 4.1.3.3), which catalyzes the cleavage of *N*-acetylneuraminic acid (sialic acid, **1a**) to *N*-acetylmannosamine (ManNAc, **2a**), and sodium pyruvate **3** (Scheme 2). In



2a R^1 = NHAc, R^2 = CH_2OH **1a** R^1 = NHAc, R^2 = CH_2OH
2b R^1 = OH, R^2 = CH_2OH **1b** R^1 = OH, R^2 = CH_2OH
2c R^1 = OH, R^2 = H **1c** R^1 = OH, R^2 = H

Scheme 2. NANA aldolase catalyzes the cleavage of sialic acid **1a** to ManNAc **2a** and sodium pyruvate **3**; in the presence of excess sodium pyruvate, aldol products **1a–c** are generated from the respective substrates **2a–c**.

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